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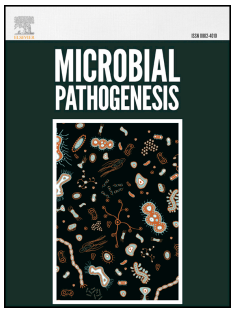

Evaluation the cytotoxic effect of cytotoxin-producing *Klebsiella oxytoca* isolates on the HEp-2 cell line by MTT assay

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Evaluation the cytotoxic effect of cytotoxin-producing *Klebsiella oxytoca* isolates on the HEp-2 cell line by MTT assay

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Abstract

Background: The cytotoxic effects on epithelial cells of the human are not observed in other strains of *Klebsiella* spp and are only observed in *K. oxytoca* strains. MTT assay was used to evaluate cytotoxic activity. In this study, colorimetric method was used to evaluate the cytotoxic effect of cytotoxin-producing isolates on Hep-2 cell line and determines the percentage of surviving cells.

Materials and methods: In this study, we collected a total of 75 K. oxytoca strains isolate and we detected the production of toxins and their cytotoxic effects on HEp-2 cells. Colorimetric method such as MTT assay was used to evaluate the cytotoxic effect of cytotoxin-producing isolates on Hep-2 cell line and determines the percentage of surviving cells.

Results: Nine isolates had cytotoxic effects on HEp-2 cells. The results of MTT assay showed that the isolated strains were different from the control stain in terms of toxinogenicity and cytotoxic effects on HEp-2 cells at the studied dilutions (1:3, 1:6, 1:12, 1:24, 1:48, and 1:96).

Conclusions: In the current study, Percentage of Hep-2 surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions of cytotoxin-producing *Klebsiella oxytoca* isolates was different.

Keywords: cytotoxic effect , cytotoxin, *Klebsiella oxytoca*, HEp-2 cell, MTT assay

1. Introduction

K. oxytoca , as an opportunistic pathogen, produces cytotoxin, which results in peritoneal colon destruction. Cytotoxin is one of the pathogenic factors of this bacterium, and one of the important factors involved in the virulence of the bacterium [1, 2] ; it is also associated with the pathogenicity of *K. oxytoca*. This bacterium is the etiologic factor of hemorrhagic colitis which is associated to the use of antibiotics in adults and the elderly [3].

Studies have shown that *K. oxytoca* can demonstrate its pathogenesis via producing cytotoxin in human. *K. oxytoca* strains associated with the colitis, and mucosal skin infections in humans are able to produce cytotoxin, which may be somewhat due to the pathogenesis of this bacterium [4]. The feature of this cytotoxin was introduced in 1989 and 1992, when it observed that this bacterium could round and kill the cell lines of HEp-2, HeLa, CHO, and Vero in vitro [5, 6]. In addition to *K. oxytoca* strains, *Micrococcus* and *Streptomyces* strains are also able to produce Tilivalline cytotoxin [7]. Tilivalline is the only pyrrolobenzodiazepine (PBD) that is produced in the microbiota of human gut and can disturb the intestinal epithelial barrier via the induction of apoptosis and reduction of epithelial resistance [1]. Cytotoxin is one of the important pathogenic factors, which plays a role in the virulence of this bacterium. Cytotoxin is associated with the pathogenesis of *K. oxytoca* [3, 8]. The cytotoxic effects on epithelial cells of the human are not observed in other strains of *Klebsiella* spp and are only observed in *K. oxytoca* strains. This study aimed to evaluation the cytotoxic effect of cytotoxin-producing *Klebsiella oxytoca* isolates on the HEp-2 cell line by MTT assay.

2. Material and Methods

2.1. Bacterial isolates

A total of 75 *K. oxytoca* strains were collected from several hospitals(Shariati, Imam Khomeini, Imam Hossinn hospitals and noor laboratory) in Tehran between 2015 and 2016. Clinical strains were isolated from stool, blood, urine, sputum, and wounds. *K. oxytoca* strains were isolated from 3750 clinical samples, including stool, blood, urine, sputum, and wounds.

2.2. Verification of K. oxytoca isolates

All bacterial strains which underwent standard microbiological tests such as oxidase test; glucose and lactose fermentation, gas and H_2S production; motility and indole production; citrate utilization; urease production; methyl red (MR) and Voges-Proskauer(VP) tests in microbiology laboratories and were detected as *K. oxytoca* strains were also detected and verified by PCR through amplification of Polygalacturonase specific gene (*pehX*) [9, 10, 11].

2.3. Cytotoxin production assay

Production of toxin determined by cytotoxic effects on HEp-2 cells. The cytotoxicity assays of *K. oxytoca* strains were performed using previously described methods [10, 12]. In brief, HEP-2 cell line (ATCC CCL-23) was used for screening this cytotoxin. A 1:1 dilution of the filtered supernatant from culture of *K. oxytoca* strains with PBS was added to the each well of a 96-well plate was seeded with $1x10^5$ HEp-2 cells and inoculated onto HEp-2 cells followed by incubation in 5% carbon dioxide at 37 ° C for 72 hours. A positive cytotoxic effect was recorded as cell rounding under light microscopy. The positive control of cytotoxin-Producing *K. oxytoca* MH43-1 was a gift from Dr Christoph Hoegenauer, Department of Internal Medicine, Medical University of Graz, Austria. The *K. oxytoca* strain ATCC 13182 served as negative control.

2.4. MTT assay

MTT assay (Sigma-Aldrich, USA) was used to evaluate cytotoxic activity. In this study, colorimetric method was used to evaluate the cytotoxic effect of cytotoxin-producing isolates on Hep-2 cell line and determines the percentage of surviving cells. The MTT assay uses the

power of the mitochondrial succinate dehydrogenase enzyme to convert the yellow salt of MTT to the purple formazan crystalline which is insoluble in water. After the addition of DMSO (Merck, Germany) detergent solvent, it is solved and the produced color is measured at the specified wavelength by using an ELISA reader. To do this process, HEp-2 cells are passaged in an RPMI 1640 medium (Caisson, USA) containing 10% fetal bovine serum (Invitrogen, USA) and 100 u/ml penicillin and 100 µg/ml streptomycin. The trypsin-treated cells with a concentration of 1.5×10^4 cells were cultured in each 96-well plate (Biofil, China) with a volume of 100 µl. Then, various dilutions of the supernatant (1:3, 1:6, 1:12, 1:24, 1:48, and 1:96) of the cytotoxin, producing bacteria were added to the wells, and HEp-2 cells were incubated at 37 ° C with 5% carbon dioxide for 48 hours. Afterward, the cells were then examined for cytotoxic effects (cell rounding). In the next step, the wells were washed with 200 µl of PBS and then 200 µl of PBS and 25 µl of MTT solution was added to the wells and incubated for 1 hour at 37 ° C. Subsequently, optical absorption was recorded by ELISA reader (STAT FAX, USA) at a wavelength of 595 nm. The percentage of surviving cells was calculated and determined using the following formula. For each dilution, the test was repeated three times, and the results were recorded [4, 13].

Percentage of surviving cells = Mean optical absorption of cells exposed to the supernatant / Mean optical absorption of control cells × 100.

Based on the results of One-way ANOVA, the test groups were compared to the control group. The significance of the data was determined by SPSS software Ver. 22 through using one-way ANOVA and Donnett tests at a significance level of $P < 0.05$.

3. Results

Of all the isolates, five *K. oxytoca* strains isolated from the stool cultures, two strains isolated from the blood cultures, one strain isolated from the wound cultures, and one strain isolated from the urine cultures had cytotoxic effects on HEp-2 cells. The strains isolated from sputum cultures had no cytotoxic effects on HEp-2 cells.

3.1 *MTT results*

Fig. 1 to 9 present the results of the tests determining the percentage of surviving cells exposed to different dilutions prepared from the supernatant of the cytotoxin producing *Klebsiella oxytoca* isolates, *K. oxytoca* MH43-1 strain (cytotoxin producing strain) and *K. oxytoca* 13182 strain (cytotoxin-negative) together with HEp-2 cells. (Dilution 1: 1:3

Supernatant dilution, Dilution 2: 1:6 Supernatant dilution, Dilution 3: 1:12 Supernatant dilution, Dilution 4: 1:24 Supernatant dilution, Dilution 5: 1:48 Supernatant dilution, Dilution 6: 1:96 Supernatant dilution, *K. oxytoca* MH43-1 , K.o 13182: *K. oxytoca* 13182)

1:3 supernatant dilution:

The mean optical absorption of HEp-2 cells exposed to 1:3 supernatant dilution of K.o 1-9 isolates and *K. oxytoca* MH43-1 strain and *K. oxytoca* 13182 strain had a significant difference with the mean optical absorption of HEp-2 cells exposed to 1:3 control dilution ($P < 0.05$).

1*:6 supernatant dilution***:**

The mean optical absorption of HEp-2 cells exposed to 1:6 supernatant dilution of K.o 1-9 isolates and *K. oxytoca* MH43-1 strain and *K. oxytoca* 13182 strain had a significant difference with the mean optical absorption of HEp-2 cells exposed to 1:6 control dilution ($P < 0.05$).

1:12 supernatant dilution:

The mean optical absorption of HEp-2 cells exposed to 1:12 supernatant dilution of K.o 1-9 isolates and *K. oxytoca* MH43-1 strain had a significant difference with the mean optical absorption of HEp-2 cells exposed to 1:12 control dilution ($P < 0.05$). The mean optical absorption of HEp-2 cells exposed to 1:12 supernatant dilution of *K. oxytoca* 13182 strain did not have a significant difference with the mean optical absorption of HEp-2 cells exposed to 1:12 control dilution ($P > 0.05$).

1:24 supernatant dilution:

The mean optical absorption of HEp-2 cells exposed to 1:24 supernatant dilution of K.o 1-9 isolates and *K. oxytoca* MH43-1 strain had a significant difference with the mean optical absorption of HEp-2 cells exposed to 1:24 control dilution ($P < 0.05$). The mean optical absorption of HEp-2 cells exposed to 1:24 supernatant dilution of *K. oxytoca* 13182 strain did not have a significant difference with the mean optical absorption of HEp-2 cells exposed to 1:24 control dilution ($P > 0.05$).

1:48 supernatant dilution:

The mean optical absorption of HEp-2 cells exposed to 1:48 supernatant dilution of K.o 1-9 isolates had a significant difference with the mean optical absorption of HEp-2 cells exposed to 1:48 control dilution ($P < 0.05$). The mean optical absorption of HEp-2 cells exposed to 1:48 supernatant dilution of *K. oxytoca* MH43-1 strain and *K. oxytoca* 13182 strain did not have a significant difference with the mean optical absorption of HEp-2 cells exposed to 1:48 control dilution ($P > 0.05$).

1:96 supernatant dilution:

The mean optical absorption of HEp-2 cells exposed to 1:96 supernatant dilution of K.o 1-9 isolates had a significant difference with the mean optical absorption of HEp-2 cells exposed to 1:96 control dilution ($P < 0.05$). The mean optical absorption of HEp-2 cells exposed to 1:96 supernatant dilution of *K. oxytoca* MH43-1 strain and *K. oxytoca* 13182 strain did not have a significant difference with the mean optical absorption of HEp-2 cells exposed to 1:96 control dilution ($P > 0.05$).

Based on the results shown in Fig. 1 to 9, the highest percentage of surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilution, respectively, were related to K.o 2 (Fig. 2) isolate, K.o 9 isolate (Fig. 9), K.o 9 isolate, K.o 6 isolate (Fig. 6), K.o 2 isolate, and K.o 1 isolate (Fig. 1).

The lowest percentage of surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilution, respectively, were related to K.o 4 isolate (Fig. 4), K.o 6 isolate (Fig. 6), K.o 4 isolate, K.o 8 isolate (Fig. 8), K.o 1,8 isolate, and K.o 7 isolate (Fig. 7).

Based on the figures presenting the percentage of Hep-2 surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions of cytotoxin-producing isolates, at 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions, respectively, *K. oxytoca* 4 (Fig. 4) , *K. oxytoca* 6 (Fig. 6) , *K. oxytoca* 4 (Fig. 4) , *K. oxytoca* 8 (Fig. 8), *K. oxytoca* 1(Fig. 1) and 8, and *K. oxytoca* 7 (Fig. 7) were the most powerful in producing cytotoxin and had the strongest toxigenic effects.

However, at 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions, respectively, *K. oxytoca* 2 (Fig. 2), *K. oxytoca* 9 (Fig. 9), *K. oxytoca* 9, *K. oxytoca* 6 (Fig. 6), *K. oxytoca* 2

(Fig. 2), and *K. oxytoca* 1 (Fig. 1) were the least powerful in producing cytotoxin and had the weakest toxigenic effects.

The results of MTT assay showed that the isolated strains were different from the control stain in terms of toxinogenicity and cytotoxic effects on HEp-2 cells at the studied dilutions (the lowest percentage of surviving cells exposed to 1:3 supernatant dilution was related to K.o 4 isolate (Fig. 4) and the highest percentage of surviving cells exposed to 1:96 was related to K.o1 isolate (Fig. 1).

4. Discussion

K. oxytoca isolated from patients with Septicemia, bacteremia, septic arthritis, soft tissue infection, cholecystitis, urinary tract infection and AHHC [1]. In a study by Joaining et al. in 2009 in Austria, 80 strains of *Klebsiella oxytoca* isolated from clinical samples were investigated in terms of the production of cytotoxin on HEp-2 cell culture. In total, 31 isolated strains had cytotoxic effects on HEp-2 cell culture. Using MTT assay, the researchers investigated the percentage of the survival of HEp-2 cells exposed to 1:3, 1:12, 1:48, 1:192, and 1:768 supernatant dilutions of the cytotoxin-producing isolates. The results of MTT assay (which was used to investigate the cytotoxic effects) showed that at 1:3 dilution 11 isolates had low cytotoxin production capacity, at 1:12 dilution 14 isolates had moderate cytotoxin production capacity, and at 1:48 dilution 6 isolates had high cytotoxin production capacity [4].

In a study by Ulbi et al. in 2012 in Austria, the researcher studied 20 patients with AAHC who had been diagnosed between 1996 and 2008. Using the two methods of API20E and PCR, 9 isolate of *Klebsiella oxytoca* were identified. After conducting an assay for cytotoxic activity on Hep-2 cell culture, because of the production of cytotoxin, the cytotoxic effects on HEp-2 cell culture was reported for 6 isolates of *Klebsiella oxytoca*. Using the MTT assay, the percentage of the survival of HEp-2 cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions of cytotoxin producing isolate supernatant was evaluated and the MTT assay was positive in more than 50% of the patients. Two isolate at 1:3 and 1:24 dilutions had cytotoxin producing capacity and toxicogenic effects. One of the *Klebsiella oxytoca* isolates at 1:48 dilution, like 1:6 dilution, had cytotoxin producing capacity and high toxicogenic effects. The figures presenting the percentage of the survival of HEp-2 cells exposed to dilution 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions for the six cytotoxin producing isolate showed the lowest percentage of surviving cells exposed to 1:3 supernatant

dilution was related to the two isolated *Klebsiella oxytoca* strains and the highest percentage of surviving cells exposed to 1:96 supernatant dilution was related to a *Klebsiella oxytoca* strain isolated from the stool of a patient with AAHC [15]. The results of MTT assay reported by the mentioned study are consistent with our results; however, in Ulbi et al.'s study the *Klebsiella oxytoca* strains were isolated from the stool of patients with AAHC, while in our study, the *Klebsiella oxytoca* strains were isolated from clinical samples of the patients (stool, urine, blood, wounds, and sputum). In our study, isolated strains were different in terms of toxinogenicity and cytotoxic effects on HEp-2 cells at the 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions.

Conflict of interest

The authors declare that there was no conflicts of interest

Acknowledgments

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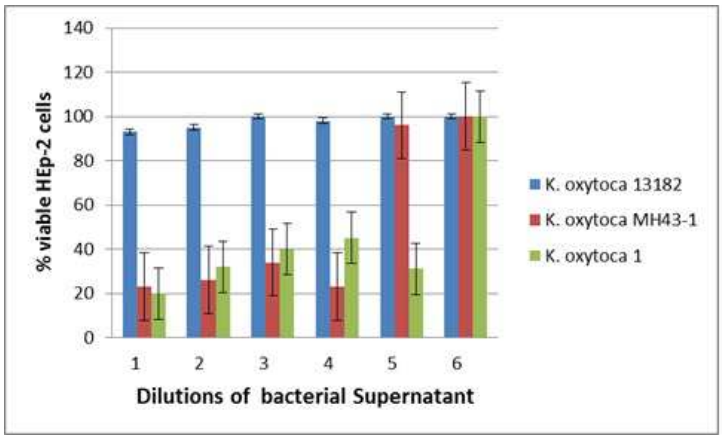

Fig. 1. Percentage of Hep-2 surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions of isolates of *K. oxytoca* 1, *K. oxytoca* MH43-1 strain, and *K. oxytoca* 13182 strain

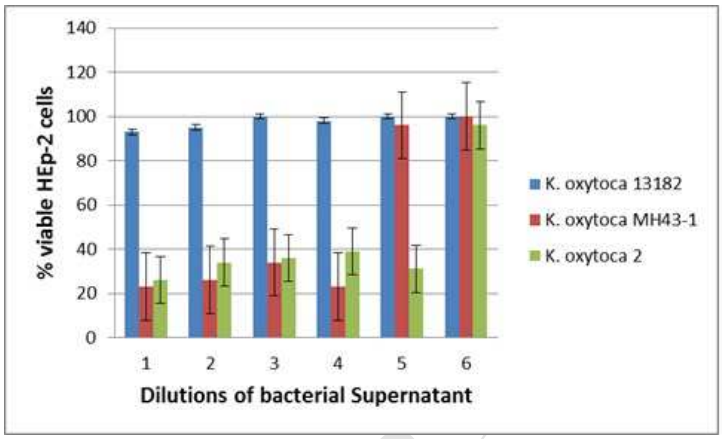

Fig. 2. Percentage of Hep-2 surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions of isolates of *K. oxytoca* 2, *K. oxytoca* MH43-1 strain, and *K. oxytoca* 13182 strain

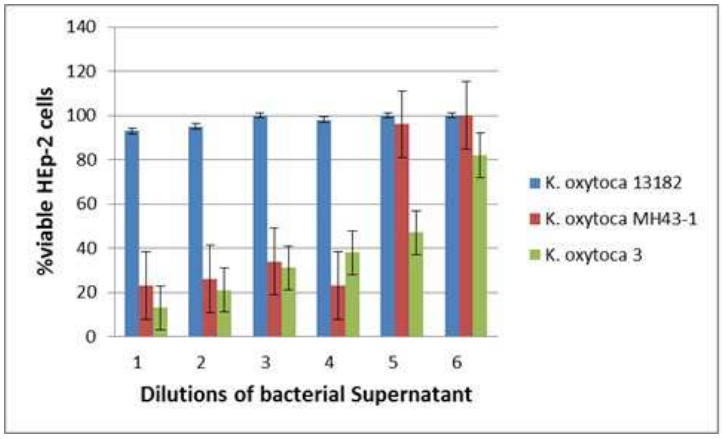

Fig. 3. Percentage of Hep-2 surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions of isolates of *K. oxytoca* 3, *K. oxytoca* MH43-1 strain, and *K. oxytoca* 13182 strain

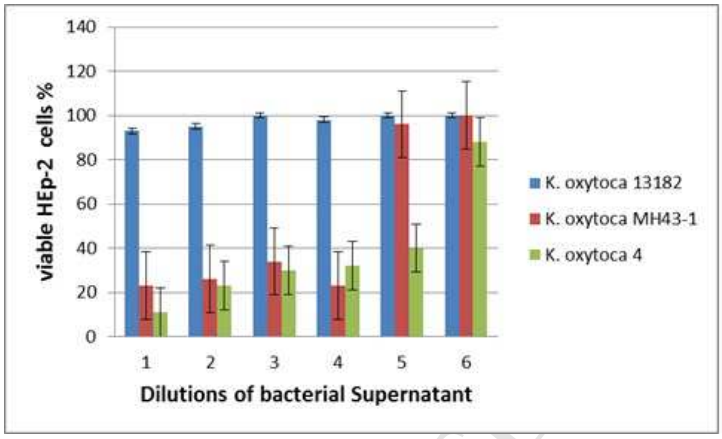

Fig. 4. Percentage of Hep-2 surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions of isolates of *K. oxytoca* 4, *K. oxytoca* MH43-1 strain, and *K. oxytoca* 13182 strain

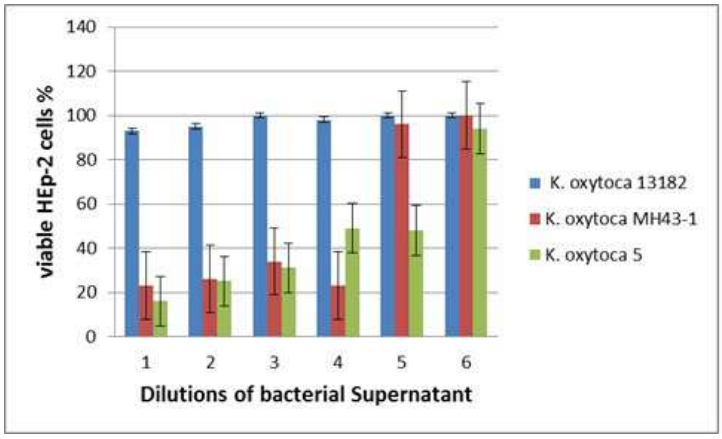

Fig. 5. Percentage of Hep-2 surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions of isolates of *K. oxytoca* 5, *K. oxytoca* MH43-1 strain, and *K. oxytoca* 13182 strain

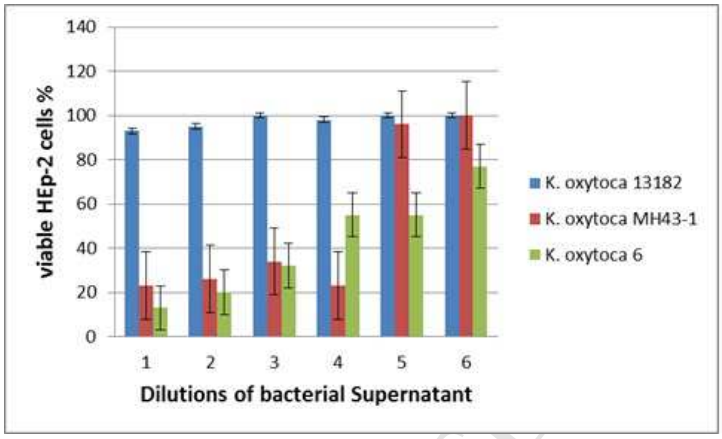

Fig. 6. Percentage of Hep-2 surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions of isolates of *K. oxytoca* 6, *K. oxytoca* MH43-1 strain, and *K. oxytoca* 13182 strain

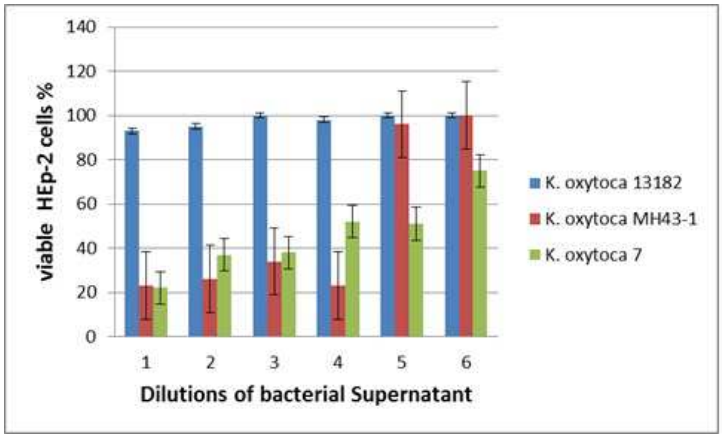

Fig. 7. Percentage of Hep-2 surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions of isolates of *K. oxytoca* 7, *K. oxytoca* MH43-1 strain, and *K. oxytoca* 13182 strain

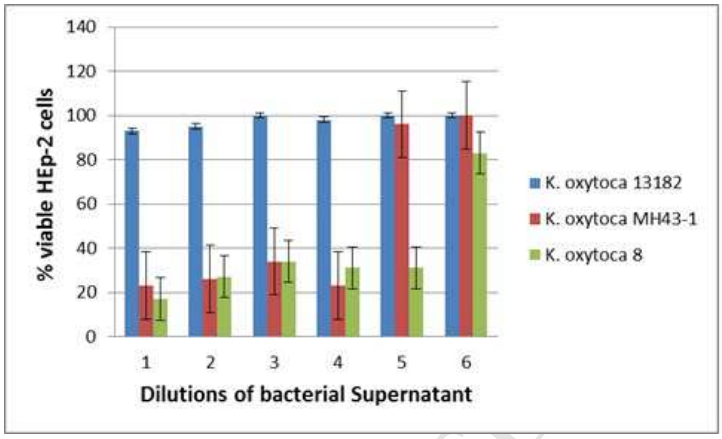

Fig. 8. Percentage of Hep-2 surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions of isolates of *K. oxytoca* 8, *K. oxytoca* MH43-1 strain, and *K. oxytoca* 13182 strain

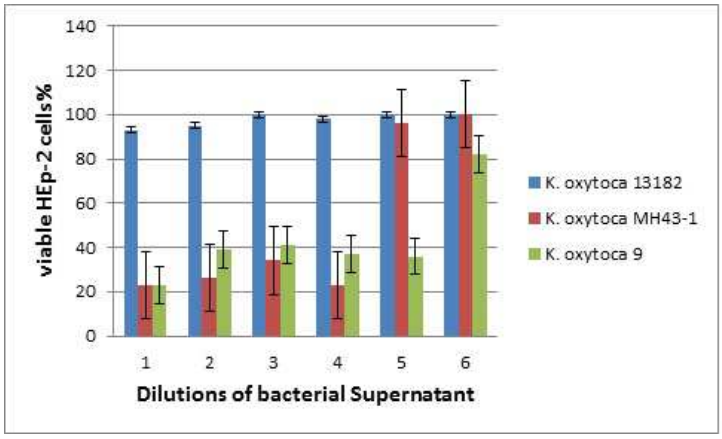

Fig. 9. Percentage of Hep-2 surviving cells exposed to 1:3, 1:6, 1:12, 1:24, 1:48, and 1:96 supernatant dilutions of isolates of *K. oxytoca* 9, *K. oxytoca* MH43-1 strain, and *K. oxytoca* 13182 strain

Nine *K. oxytoca* isolates had cytotoxic effects on HEp-2 cells.

MTT assay was used to evaluate the cytotoxic effect of cytotoxin on Hep-2 cells.

MTT assay was used to determines the percentage of surviving cells.

Isolated strains were different in terms of toxinogenicity and cytotoxic effects on HEp-2 cells.

Percentage of Hep-2 surviving cells exposed to supernatant dilutions of cytotoxin was different.